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ATM activates p53 by regulating MDM2 oligomerization and E3 processivity

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 24 June 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three reviewers, whose comments are attached below. As you will see, all referees find your study on ATM-dependent activation of p53 via phosphorylation and activity modulation of Mdm2 potentially interesting and important. However, while they all appreciate the results on ATM phosphorylation of Mdm2 and its effect on p53 stability, it is apparent that they are less convinced that your present set of data has also conclusively established how Mdm2 phosphorylation may molecularly affect Mdm2-dependent ubiquitination, pointing out both internal inconsistencies as well as discrepancies with published observations.

Given the potential importance of your primary observations and the overall interest expressed by the referees, I am inclined to give you the opportunity to respond to the referees' criticisms through a revised version of the manuscript. However, I hope you understand that we are currently not in a position to make strong commitments with regard to publication of a revised manuscript, as it is presently not clear if the requested further work will be able to clarify the inconsistencies and validate the main conclusions, or rather confound them. Still, should you feel confident that you might be able to address the key criticisms and to convince the critical referees, we should be happy to consider a revised manuscript further for publication. In this respect, please bear in mind that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your

revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript presents a study aimed at defining the mechanism by which the protein kinase ATM activates p53. The focus of the study is the ubiquitin E3 ligase, Mdm2, which is known to be responsible for regulating the levels of p53 protein via its ability to generate poly-ubiquitin chains on p53. There are N main findings reported in this paper: 1) the identities of six novel phosphorylation sites within the region of Mdm2 that is just N-terminal to its C-terminal RING domain, 2) ATM-dependent phosphorylation of several of the identified sites after DNA damage, 3) an inverse relationship between Mdm2 phosphorylation and p53 poly-ubiquitination, 4) phosphorylation of Mdm2 (or use of a phospho-mimic mutant) disrupts interaction between Mdm2 RING domains. The authors use their results in support of a model in which control of the oligomerization state of Mdm2 by phosphorylation that occurs in response to DNA damage inhibits the E3 ligase activity of Mdm2, thereby stabilizing p53. While this is an attractive model, not all the conclusions are well supported by data presented in the manuscript and supplemental figures. In particular, neither the conclusion that phosphorylated Mdm2 monoubiquitinates p53 nor that phosphorylated Mdm2 disrupts Mdm2 oligomerization are rigorously shown (see detailed comments below.) As these two conclusions comprise important aspects of the ultimate model proposed, it is critical that the relevant data and their interpretation be as sound as possible. If that can be accomplished, the paper should be of considerable interest to a large readership, including those interested in p53 regulation and in protein ubiquitination pathways and the mechanisms in general.

Specific Comments.

1. The authors claim that Mdm2-6D mainly mono-ubiquitinates (or multiply mono-ubiquitinates) p53. It is not clear upon what they base this claim. Oddly, the gels in Figure 4b are quite smeared, so a clear, specific band for the species monoUb-p53 is not evident. Furthermore, to draw a rigorous conclusion regarding mono-ubiquitination, assays must be done in which either methylated-Ub and the lysine-less mutant of Ub (Ub-K0) is used.

2. The gel filtration profiles presented in Figure 5c and Supp. Figure 3 are perplexing. The Mdm2 IPed from "control" SJSA cells appears only in a very large complex (~200kDa) or as a monomer. The recombinant analog of that experiment shows Mdm2 only in the large species (this may be an artifact of the GST-tag, which is itself dimeric.) The Mdm2 IPed following IR is almost completely monomeric, although it seems highly unlikely that all Mdm2 in the cell has been phosphorylated. The recombinant analog to this experiment (Mdm2-6D) elutes in a continuum, indicative of a disperse. heterogeneous population of species. Finally, the Mdm2 IPed from cells treated with IR in the presence of a kinase inhibitor is mostly found in large species (although spread over a much wider range. Furthermore, that protein is also found throughout the elution profile, giving a pattern that is much more similar to the Mdm2-6D mutant. It is not clear that the recombinant Mdm2 proteins were assays for their E3 ligase activity, so there is no way to know if they are folded into an active conformation.

Minor Comments.

1. Figure 1c: label "PS4386" cannot be correct.

Referee #2 (Remarks to the Author):

The manuscript by Cheng et al., 'ATM activates p53 by regulating MDM2 oligomerization en E3 processivity', describes a new mechanism by which p53 is stabilized upon ionizing radiation. The authors start with identifying phosphorylation sites in Mdm2 upon ionizing radiation. Interestingly, in addition to the described sites within the C-terminal RING domain region, S395 and S407, the authors find 4 new sites, S386, T419, S425 and S429. Two of these have the typical ATM target sequence, which means that the phosphorylation site is followed by a Q.
Further experiments are focused on the S386 and S429 sites, because against these sites phosphor-specific antibodies were raised. Subsequent experiments clearly show that these sites are bona fide phosphorylation sites, which are phosphorylated upon treatment of cells with various DNA damaging agents, and that upon IR the phosphorylation is ATM dependent and these sites are direct ATM targets. With the use of mutants in which one or more of the six phosphorylation sites are altered into phospho-mimicking amino acids, it was found that mutating just one of these sites is sufficient to inhibit the degradation of p53 by Mdm2. Mutating all does not appear to have an additional effect. On the other hand, such mutations do not appear to affect the degradation of Mdmx by Mdm2, and also not the ubiquitination of Mdm2 itself. These data, in general, look very good although I have some minor comments on a few figures.
Subsequently, the authors try to elucidate the mechanism by which the phosphorylation might inhibit the p53 degradation. To that end, they performed in vivo Ub assays, initially with the use of His6-tagged Ubiquitin. In this assay, they find no significant difference between WT and 6D Mdm2, while the 6A might be slightly skewed towards higher MW ubiquitinated p53 species. However, the authors argue that this assay preferentially detects the most abundant Ub-p53 species, which are the (multiple) mono-Ub species. I do not agree. Every species of p53 will be detected with equal sensitivity. In these transfection assays indeed most of Ub-p53 species are multiple mono-Ub, but to prove that all the species the authors detect, they should have used a Ubiquitin that is mutated in its lysines so cannot form chains anymore. Then, they use a Myc-tagged Ubiquitin, and probe the blot with ant-Myc upon p53 IP. In this way they preferentially detect p53 species containing multiple Ub molecules, I agree with that. However, it still does not explain the huge difference now seen between the WT and 6D mutant (figure 4A). In the supplemental figure 2 there is no real difference in intensity in the bands between the 72 and 95 kDa marker with use of WT or 6D, while with 6A intensity if even lower. Therefore, around that size there should also be no difference between WT and 6D in figure 4A, and 6A should be lower. This is clearly not what is seen. The authors should explain. Better, an experiment as shown in figure 4A should be done, but duplicate blots should be incubated with either anti-Myc or anti-p53. That would better solve this issue.
Also the data shown in figure 4B are not very convincing. The 6A mutant appears to be better than WT and 6D, but hardly difference between 6A and 6D. CIP activates activity of WT, but also of 6D to some extent. If the amount of unmodified p53 remaining is also a measure of how much is ubiquitinated, than after CIP hardly a difference is seen between WT and 6D, neither before nor after CIP treatment, while this is clearly less after use of 6A in absence of CIP. So, the data indicating that phosphorylation of Mdm2 in the C-ter RING domain specifically inhibits poly-Ub of p53 are not convincing to me.
To find an explanation for their observation, the authors subsequently start to investigate the oligomerization of Mdm2, based upon an earlier publication that Mdm2 multimers are more activity as an E3 ligase. Indeed, interestingly, the authors find that IR shifts the gel filtration profile of the Mdm2 RING towards monomers, which is inhibited by the ATM inhibitor. This is supported by the fact that the 386E mutant of Mdm2 does hardly form multimers after transfection into H1299 cells. However, bacterially produced Mdm2-6D protein is indeed showing more monomers than WT, but still the majority is in multimers. I wonder whether the authors have an explanation for this observation. Does it mean that in vivo not only the phosphorylations of the RING affects the oligomerization of Mdm2 but that also other -ATM-dependent- regulatory mechanisms play a role?
In a follow up experiment, the authors make use of the fact that unphosphorylated and phosphorylated Mdm2-RING have a different apparent MW on SDS-PAGE. IP'ing full length Mdm2 results in a better co-IP of unphosphorylated versus phosphorylated Mdm2 RING. The result in figure 6D is not very well interpretable, since in the input also the amount of P-RING is lower than unP-RING. It would be more convincing if the authors could provide a quantification of the ratio between these two RING species in input and after IP.
Even so, assuming that indeed the phosphor-Mdm2 RING cannot homo-dimerize, it still can bind to the RING of Mdmx. Based upon publications from the Vousden and the Yuan lab, one would expect that such a heterodimer would be perfectly able to ubiquitinate and degrade p53, maybe even better than an Mdm2 homo-oligomer. I am very surprised that he authors not even discuss these

publications. In addition, the publication that is mentioned by the authors, by the group of Prives on the Mdm2 oligomerization, shows that a monomeric RING has maybe less but still a very significant activity as an E3 ligase. Also this does not fit with the results mentioned in this manuscript. Furthermore, it has been shown by e.g. Lane's group, Steven Grossman and Christine Blattner that ubiquitination of p53 by Mdm2 might not be sufficient for degradation but that possibly post-Ub steps are involved, e.g. targeting to the proteasome. Since I find the results on the ubiquitination of p53 by the Mdm2 6D mutant not very convincing, why not look into that direction?
Lastly, the authors present a number of experiments with U2OS derived stable transfectants of Mdm2 and Mdm2-6A. Whether levels as in SJSA are physiologically relevant is questionable, since in this cell line expression of Mdm2 is extremely high. Could the authors give estimation how much the Mdm2 levels are increased compared to parental U2OS? Are the increased levels of Mdm2 affecting basal levels of p53 and of Mdmx, and maybe of p21 since that also has been shown to be a target of Ub-ligase activity of Mdm2? And if so, is the effect same in U2OS-WT and U2OS-6A cells? I feel these questions have to be answered to be able to interpret the results shown in figure 7 and figure 8. Even so, I agree that in 6A cells p53 stabilization appears to be delayed, and p21 induction is strongly reduced upon IR.
With NCS the distinct effect on p21 induction appears to be much less, when comparing U2OS-Mdm2 and U2OS-6A. Also in U2OS-Mdm2 the induction of p21 is much less than in parental U2OS by NCS. Why this difference in behavior after IR and NCS? I am also surprised that ActD is not showing a distinct effect between Mdm2 WT and 6A, since several publications have shown that ActD treatment can lead to S15 phosphorylation of p53. In accordance with lower p21 induction, the cell cycle arrest as measured by H3-Thymidine incorporation is delayed in the 6A U2OS cells. What surprises me somewhat is that at 24 hrs the difference between WT and 6A has almost disappeared, while in figure 7 still at 24 hrs no induction of p21 is seen. Is this a p53/p21- independent effect upon IR?
It should be mentioned that the fact that phosphorylation of Mdm2 by an ATM-dependent manner is needed for full p53 activation has been shown earlier quite clearly by Stommel and Wahl, so the experiments shown in figures 7 and 8 are not really providing more insight into regulation of p53-Mdm2 upon DNA damage.

The authors argue in the Discussion section that they do not see a transient reduction of Mdm2 levels upon IR, but that this original observation might be an artifact of the use of the SMP14 antibody. Although possibly outside of the scope of this manuscript, this is an important observation that would shed new light on earlier publications. Therefore, either the authors should show this distinct effect of SMP14 (with the use of the parental U2OS cells, for example), or it would be better not to mention it at all.

Minor/specific comments:
Figure 1C: typo in labeling (it says PS4386). In legends it says that cells are treated with 10 Gy for 4 hrs. I think that is not completely correct. Cells are irradiated with 10 Gy, and after 4 hrs they are harvested. Similar descriptions are found elsewhere in the legends.
Figure 2A: what is the meaning of the His6-Mdm2 in this figure/experiment? Either mention it somewhere or leave it out.
Figure 2C: I would have expected that 2 hrs after IR levels of p53 and Mdm2 are already increased to some extent, based also upon figure 2a. Why is that not seen here? Or, were the cells treated with MG132?
Figure 3a: the authors say that the phosphor-mimicking mutants of Mdm2 are 'deficient' in degradation of p53. Maybe this is a bit too strongly put, since they clearly show still quite an activity. It would be nice if the authors would have quantified figure 3a, or even better would have shown a titration experiment with the mutants and wt side-by-side.
As mentioned above, an experiment as shown in figure 4a should be repeated, and probed not only with anti-Myc but also with anti-p53. Theoretically the last incubation should give a result as in the His-Ub pull-down, but the whole experiment is somewhat different, so should be tested.
Supplemental figure 1: Self-Ub of Mdm2: I would say that the 6D is more active than wild-type. Is this seen consistently?
Figure 6: the 4B11 antibody has its epitope in the RING domain. Is the excluded that phosphorylation of the RING on one or more sites could weaken the interaction with 4B11, so that actually the amount of P-RING is underestimated?

Referee #3 (Remarks to the Author):

In their manuscript, the authors suggest that ATM stabilizes p53 in response to IR by phosphorylating several amino acids in the C-terminal part of Mdm2 which reduce Mdm2 oligomerization via the Ring domain.
To support their conclusions, the authors showed that two (S386; S429) of 6 phosphorylation sites identified by mass spec are phosphorylated in vivo by using phosphorylation specific antibodies. ATM can phosphorylate these sites in vitro and phosphorylation is absent in ATM-negative cells or after ATM inhibition. Replacement of the phosphorylated serines with acidic amino acids prevented p53 degradation. While replacing the 6 terminal serines with an alanine enhanced p53 ubiqutiylation, and reduced the accumulation of p53 after IR after ectopic expression, substituting them with an aspartic acid prevented p53 ubiquitylation. The authors furthermore argue that phosphorylation of the C-terminus of Mdm2 prevents RING domain oligomerization.

This is indeed an elegant study with a number of clever experiments to resolve a complex issue. However, sophisticated experiments also bear the intrinsic risk of creating artificial models and the regulation of p53 appears to be particularly prone to artefacts due to results of cell culture experiments that cannot be reproduced e.g. in animal models. Therefore, these studies need to be evaluated particularly carefully.

Intriguingly, the results from Chen at al., as beautiful and consistent as they are, do not not comply with several previous and important observations.
1) Lu and Lane reported in 1993 that the kinetics of p53 in response to UV and ionising irradiation differs, a finding that has been reproduced by several investigators in the meantime. Cheng et al., though, show that serine 386 and serine 429 of Mdm2 are phosphorylated at the same time and to the same extend (Figure 1d), suggesting that phosphorylation of these sites does not differe between UV and IR.
2) Maki and Howley showed in 1997 that in contrast to UV-irradiation, p53 that accumulates after IR is still ubiquitylated, a finding that is, moreover, consistent with the different kinetics of p53 accumulation that are most likely due to different mechanisms. In contrast, Chen et al., report that ATM phosphorylation of Mdm2 prevents p53 polyubiquitylation.
3) Boehme et al., have shown in 2008 that p53 accumulates independent of ATM, at least in some cell lines. Cheng et al., though suggest that the accumulation of p53 in response to Mdm2-RING phosphorylation is caused by the ATM kinase.
Since these previous observations have been derived from non-manipulated cells they do not bear such a high risk of false results as observations that are derived upon manipulating the system by transfections and mutagenesis. These issues therefore need to be carefully addressed.

Additional comments:
- 1) figure 3a: what is the reason why one serine was replaced with aspartic acid while the other one was replaced with glutamic acid? How would the result look if serine 386 would be replaced with aspartic acid and serine 429 with glutamic acid? Nevertheless, it needs to be kept in mind that replacing a serine with an acidic acid does not always mimic a phosphorylated serine.
- 2) figure 3b) Obviously, the Mdm2 mutant Mdm2-6A is more competent than wild type Mdm2 in targeting for p53 degradation. Since wild type Mdm2 was not phosphorylated in the C-terminus under normal conditions as shown for example in figure 1b, 2c or 2c, this could indicate that replacing 6 amino acids with an alanine has an i mpact on Mdm2 activity that is distinct from phosphorylation of these serines.
- 3) figure 4a) This figure shows that the 6D mutant does no longer polyubiquitylate p53. In order to distinguish between non- and mono-ubiqutiylation, it is essential to include a sample where no ubiquitin has been transfected to determine background levels.
- 4) figure 4b) Strikingly, while in figure 4a, the 6D mutant lacked any E3 activity, in figure 4b, and in supplementary figure 2a, the 6D mutant is almost as active as wild type Mdm2 in ubiquitylating p53?
- 4) figure 6c) Although most likely, there is no formal proof in this figure hat the smaller forms are indeed phosphorylated Mdm2. In addition, there is also a shift in the early fraction upon treatment with CIP.
- 5) figure 5 and Supplementary figure 3) The gel filtration experiments look vary variable. While in figure 5c, wt C-term Mdm2 fractionates as oligomers and monomers, in supplementary figure 3a,

Mdm2 is everywhere, although the reviewer agrees that the concentration is higher in earlier fractions. In contrast, in supplementary figure 3b, , Mdm2 is only in the early fractions. Taken all these different experiments into account, the 6D mutant in supplementary figure 3b did not show a clear difference to the wild type protein in supplemental figure 3a or to the Ku55933-treated and irradiated wild type Mdm2 in figure 5c. Moreover, while the single substituted serine 386 aspartic acid mutant, showed a clear difference to wild type Mdm2 in gel filtration experiments (supplementary figure 3a), the corresponding alanine mutant had no effect on p53 degradation as shown in supplementary figure 4.
- 6) supplementary figure 4: Obviously, only replacement of all 6 C-terminal serines affects Mdm2 activity, but only phosphorylation of three of them (S 386, S395, S429) is supported by additional experiments, e.g. by phosphorylation-specific experiments. Moreover, alanine replacement of these 3 serines, that have been clearly shown to be phosphorylated after IR, does not impair Mdm2 function. How sure can we be that the 6A and 6D mutants really mimick the physiologic situation?
- 7) supplementary figure 4b) How does it come that p53 levels are high at 0hr after transfection of cDNA3 and Mdm2, lower at 3hr and particularly low at 6hr after irradiation?

1st Revision - authors' response 27 July 2009

We are submitting a revised manuscript titled "ATM activates p53 by regulating MDM2 oligomerization and E3 processivity" to the EMBO Journal. The manuscript had been reviewed as EMBOJ-2009-71449. We appreciate the expert comments from the reviewers and have performed additional experiments based on their suggestions, and revised the manuscript accordingly. These will be address in detail below.

Major changes in figures

Figure 3c. New data of MDM2-6A and MDM2-6D titration (Reviewer 2).

Figure 4a. New data of p53 ubiquitination assay (Reviewer 2).

Figure 4c. New data of p53 mono ubiquitination using K0-ub (Reviewer 1 and 2).

Figure 6c. A lighter exposure of previous figure for better clarity (Reviewer 3).

Figure 6e. Quantitation of 6d (Reviewer 2).

Figure S3b. Repeat of GST-MDM2-WT and GST-MDM2-6D chromatography at lower concentration (Reviewer 1,2,3).

Revisions and clarifications (reviewer comments are underlined)

Referee #1 (Remarks to the Author):

While this is an attractive model, not all the conclusions are well supported by data presented in the manuscript and supplemental figures. In particular, neither the conclusion that phosphorylated Mdm2 monoubiquitinates p53 nor that phosphorylated Mdm2 disrupts Mdm2 oligomerization are rigorously shown (see detailed comments below.) As these two conclusions comprise important aspects of the ultimate model proposed, it is critical that the relevant data and their interpretation be as sound as possible. If that can be accomplished, the paper should be of considerable interest to a large readership, including those interested in p53 regulation and in protein ubiquitination pathways and the mechanisms in general.

Specific Comments.

1. The authors claim that Mdm2-6D mainly mono-ubiquitinates (or multiply mono-ubiquitinates) p53. It is not clear upon what they base this claim. Oddly, the gels in Figure 4b are quite smeared, so a clear, specific band for the species monoUb-p53 is not evident. Furthermore, to draw a rigorous conclusion regarding mono-ubiquitination, assays must be done in which either methylated-Ub and the lysine-less mutant of Ub (Ub-K0) is used.

Response: We have added a new figure (4c) that uses K0-Ub to show multi-mono ubiquitination products of p53. Because of the multiple lysines on p53, mono ubiquitination alone can generate products above 95 kd. MDM2-6A mainly increases products above this range, which is consistent with its enhanced poly ubiquitination activity. MDM2-6D retains significant mono ubiquitination activity as we concluded in the paper. The gels shown in Figure 4b are incubated in sodium salicylate to enhance the signals before exposure at -80∞C. It is normal that the bands are more diffused in fluorography.

2. The gel filtration profiles presented in Figure 5c and Supp. Figure 3 are perplexing. The Mdm2 IPed from "control" SJSA cells appears only in a very large complex (~200kDa) or as a monomer. The recombinant analog of that experiment shows Mdm2 only in the large species (this may be an artifact of the GST-tag, which is itself dimeric.) The Mdm2 IPed following IR is almost completely monomeric, although it seems highly unlikely that all Mdm2 in the cell has been phosphorylated. The recombinant analog to this experiment (Mdm2-6D) elutes in a continuum, indicative of a disperse. heterogeneous population of species. Finally, the Mdm2 IPed from cells treated with IR in the presence of a kinase inhibitor is mostly found in large species (although spread over a much wider range. Furthermore, that protein is also found throughout the elution profile, giving a pattern that is much more similar to the Mdm2-6D mutant. It is not clear that the recombinant Mdm2 proteins were assays for their E3 ligase activity, so there is no way to know if they are folded into an active conformation.

Response: Figure 6b suggests that IR induces ~100% phosphorylation (shifting) of MDM2. Therefore, it is not surprising that IR strongly inhibits RING oligomerization in gel filtration. As shown in Figure 1b, there is basal S386 phosphorylation in the absence of IR and in the presence of Mg132. Therefore a portion of MDM2 is expected to be monomer in untreated cells. KU55933 treatment inhibited ATM activity, therefore a complete shift to oligomer is as expected.

The original analysis of GST-MDM2-6D (Figure S3b) used high level of E. coli-produced protein, therefore favors oligomerization. We repeated the gel filtration experiment using GST-MDM2-WT and GST-MDM2-6D at 10-fold lower level (similar to cell culture) and observed more monomers of GST-MDM2-6D as expected (new Fig S3b). GST-MDM2 is less active than immunoprecipitated MDM2 from cells in ubiquitination assay (not shown).

Referee #2 (Remarks to the Author):

The manuscript by Cheng et al., 'ATM activates p53 by regulating MDM2 oligomerization en E3 processivity', describes a new mechanism by which p53 is stabilized upon ionizing radiation. The authors start with identifying phosphorylation sites in Mdm2 upon ionizing radiation. Interestingly, in addition to the described sites within the C-terminal RING domain region, S395 and S407, the authors find 4 new sites, S386, T419, S425 and S429. Two of these have the typical ATM target sequence, which means that the phosphorylation site is followed by a Q.
Further experiments are focused on the S386 and S429 sites, because against these sites phosphor-specific antibodies were raised. Subsequent experiments clearly show that these sites are bona fide phosphorylation sites, which are phosphorylated upon treatment of cells with various DNA damaging agents, and that upon IR the phosphorylation is ATM dependent and these sites are direct ATM targets. With the use of mutants in which one or more of the six phosphorylation sites are altered into phospho-mimicking amino acids, it was found that mutating just one of these sites is sufficient to inhibit the degradation of p53 by Mdm2. Mutating all does not appear to have an additional effect. On the other hand, such mutations do not appear to affect the degradation of Mdmx by Mdm2, and also not the ubiquitination of Mdm2 itself. These data, in general, look very good although I have some minor comments on a few figures.
Subsequently, the authors try to elucidate the mechanism by which the phosphorylation might inhibit the p53 degradation. To that end, they performed in vivo Ub assays, initially with the use of His6-tagged Ubiquitin. In this assay, they find no significant difference between WT and 6D Mdm2, while

the 6A might be slightly skewed towards higher MW ubiquitinated p53 species. However, the authors argue that this assay preferentially detects the most abundant Ub-p53 species, which are the (multiple) mono-Ub species. I do not agree. Every species of p53 will be detected with equal sensitivity. In these transfection assays indeed most of Ub-p53 species are multiple mono-Ub, but to prove that all the species the authors detect, they should have used a Ubiquitin that is mutated in its lysines so cannot form chains anymore. Then, they use a Myc-tagged Ubiquitin, and probe the blot with ant-Myc upon p53 IP. In this way they preferentially detect p53 species containing multiple Ub molecules,
I agree with that. However, it still does not explain the huge difference now seen between the WT and 6D mutant (figure 4A). In the supplemental figure 2 there is no real difference in intensity in the bands between the 72 and 95 kDa marker with use of WT or 6D, while with 6A intensity if even lower. Therefore, around that size there should also be no difference between WT and 6D in figure 4A, and 6A should be lower. This is clearly not what is seen. The authors should explain. Better, an experiment as shown in figure 4A should be done, but duplicate blots should be incubated with either anti-Myc or anti-p53. That would better solve this issue.
Also the data shown in figure 4B are not very convincing. The 6A mutant appears to be better than WT and 6D, but hardly difference between 6A and 6D. CIP activates activity of WT, but also of 6D to some extent. If the amount of unmodified p53 remaining is also a measure of how much is ubiquitinated, than after CIP hardly a difference is seen between WT and 6D, neither before nor after CIP treatment, while this is clearly less after use of 6A in absence of CIP. So, the data indicating that phosphorylation of Mdm2 in the C-ter RING domain specifically inhibits poly-Ub of p53 are not convincing to me.

Response: Regarding the reviewer's concern about the size range of multi-mono-ubiquitinated p53, revised Fig. 4b used K0-ub to show that we correctly defined mono-ubiquitinated p53 as those migrating below 130 kd.
The reviewer mentioned a discrepancy between Figure 4a and Figure S2a. In Figure S2a, p53 was significantly depleted by MDM2-6A, therefore even some mono ubiquitinated p53 was being consumed to make poly ubiquitinated forms. In contrast, p53 depletion was not significant in Figure 4a, therefore both low MW and high MW forms of p53 were present at high levels. We have other experiments where p53 was in excess in the His-ubiquitin assay and Wt and 6A looked more identical. Therefore, these variations are clearly related to the extent of p53 depletion in different experiments and do not affect our conclusion.
We apologize for not explaining the assays better in the manuscript. Most investigators use only one type of in vivo ubiquitination assay which is often sufficient to serve their purpose. During our study, we realized the bias of different assays and found them to be quite informative after careful consideration of the technical details. The His-ubiquitin assay uses p53 antibody so the signal is dependent on the level of ubiquitinated p53. Most of the signal in this assay is at a low MW range representative of mono ubiquitinated p53 because they are stable. The Myc-ubiquitin assay uses anti-Myc antibody and the signal is proportional to the number of Myc-ubiquitin conjugated to p53. Therefore the assay favors detection of poly ubiquitinated p53. This effect is responsible for the strong difference of 6A and 6D in Figure 4a, and small difference in Figure S2a. The in vitro ubiquitination assay has no bias because p53 was radio-labeled and the gel was dried and exposed without membrane transfer step that may lose some high MW forms. The results of in vitro assay (Figure 4b) is consistent with the in vivo results.
As to concerns about Figure 4b, we drew conclusion mostly based on the yield of high MW p53 (>130 kd). There was not a dramatic difference between Wt MDM2 and 6D because transfected MDM2 was partially phosphorylated on S386 from transfection stress according to our analysis (Figure 1c). Therefore CIP treatment has a large effect in stimulating Wt MDM2 activity. 6D was partially stimulated by CIP because it may still have other minor phosphorylation sites missed by the mass spec analysis.
As to the levels of remaining p53 in Figure 4b, they provide some indication that 6A was more active in adding the first ubiquitin thus upshifting p53. However, these reactions proceeded to the point of near substrate depletion, they were not designed to analyze the kinetics of the initial reaction (requires substrate excess). Therefore we do not believe that conclusion can be drawn based on the band intensity of remaining p53.
As suggested by the reviewer, we revised Figure 4a with both Myc and p53 blot. The results showed MDM2-6A to be more active in producing both mono and poly ubiquitinated p53, whereas 6D has significant reduction in poly ubiquitination activity (Myc blot), but retains most of the mono ubiquitination function (p53 blot).

To find an explanation for their observation, the authors subsequently start to investigate the oligomerization of Mdm2, based upon an earlier publication that Mdm2 multimers are more activity as an E3 ligase. Indeed, interestingly, the authors find that IR shifts the gel filtration profile of the Mdm2 RING towards monomers, which is inhibited by the ATM inhibitor. This is supported by the fact that the 386E mutant of Mdm2 does hardly form multimers after transfection into H1299 cells. However, bacterially produced Mdm2-6D protein is indeed showing more monomers than WT, but still the majority is in multimers. I wonder whether the authors have an explanation for this observation. Does it mean that in vivo not only the phosphorylations of the RING affects the oligomerization of Mdm2 but that also other -ATM-dependent- regulatory mechanisms play a role?

Response: As discussed in response to Reviewer 1 above, revised Fig S3b used lower GST-MDM2 levels for chromatography and showed profiles consistent with in vivo MDM2.

In a follow up experiment, the authors make use of the fact that unphosphorylated and phosphorylated Mdm2-RING have a different apparent MW on SDS-PAGE. IP'ing full length Mdm2 results in a better co-IP of unphosphorylated versus phosphorylated Mdm2 RING. The result in figure 6D is not very well interpretable, since in the input also the amount of P-RING is lower than unP-RING. It would be more convincing if the authors could provide a quantification of the ratio between these two RING species in input and after IP.

Response: As suggested, we added a quantitation of the result by densitometry which clearly shows a reduced binding of phosphorylated RING. We should point out that phosphorylation weakens but does not completely abrogate binding, since the sites are not part of the RING dimerization interface.

Even so, assuming that indeed the phosphor-Mdm2 RING cannot homo-dimerize, it still can bind to the RING of Mdmx. Based upon publications from the Vousden and the Yuan lab, one would expect that such a heterodimer would be perfectly able to ubiquitinate and degrade p53, maybe even better than an Mdm2 homo-oligomer. I am very surprised that he authors not even discuss these publications. In addition, the publication that is mentioned by the authors, by the group of Prives on the Mdm2 oligomerization, shows that a monomeric RING has maybe less but still a very significant activity as an E3 ligase. Also this does not fit with the results mentioned in this manuscript. Furthermore, it has been shown by e.g. Lane's group, Steven Grossman and Christine Blattner that ubiquitination of p53 by Mdm2 might not be sufficient for degradation but that possibly post-Ub steps are involved, e.g. targeting to the proteasome. Since I find the results on the ubiquitination of p53 by the Mdm2 6D mutant not very convincing, why not look into that direction?

Response: The biochemical data by Linke et al showed that MDM2-MDMX heterodimer has much weaker E3 activity compared to MDM2 homodimer in vitro. Furthermore, MDM2-MDMX heterodimer formed after DNA damage leads to rapid degradation of MDMX. Therefore the heterodimer is not stable and unlikely to function in the same manner as MDM2 homodimer. Animal models also ruled out MDMX as a significant regulator of p53 stability.
The Prives study showed that RING oligomers is more active than monomers although not by a large margin. This does not contradict with our model. It is important to point out that they were measuring RING self-ubiquitination activity, not p53 ubiquitination. Therefore, their result is consistent with our finding that MDM2-6D is active in self-ubiquitination in vivo (Figure S1c).
We initially considered whether MDM2-6D is defective in interaction with proteasome. We tested 6D interaction with proteasome subunit C8 in vitro and in vivo but found no difference (not shown). There is also no change in 6D self degradation. Therefore the evidence point to changes in p53 poly ubiquitination function. However, we cannot rule out an additional effect on proteasome targeting.

Lastly, the authors present a number of experiments with U2OS derived stable transfectants of Mdm2 and Mdm2-6A. Whether levels as in SJSA are physiologically relevant is questionable, since in this cell line expression of Mdm2 is extremely high. Could the authors give estimation how much the Mdm2 levels are increased compared to parental U2OS? Are the increased levels of Mdm2 affecting basal levels of p53 and of Mdmx, and maybe of p21 since that also has been shown to be a target of Ub-ligase activity of Mdm2? And if so, is the effect same in U2OS-WT and U2OS-6A cells? I feel these questions have to be answered to be able to interpret the results shown in figure 7 and figure 8. Even so, I agree that in 6A cells p53 stabilization appears to be delayed, and p21 induction

is strongly reduced upon IR.

Response: In titration analysis MDM2-6A was expressed at the same level of SJSA, which is about 10-fold higher than U2OS (not shown). As shown in Figure 7a, Wt MDM2 and 6A overexpression reduced basal p53 by ~2-fold, but did not reduce basal MDMX level. The effect on p21 basal level was also <2-fold. Our previous study also showed changing MDM2 level alone had very little effect on MDMX degradation in the absence of DNA damage signaling. The critical comparison is that when Wt MDM2 and 6A were both overexpressed, only 6A strongly repressed p53 and p21 induction by IR.

With NCS the distinct effect on p21 induction appears to be much less, when comparing U2OS-Mdm2 and U2OS-6A. Also in U2OS-Mdm2 the induction of p21 is much less than in parental U2OS by NCS. Why this difference in behavior after IR and NCS? I am also surprised that ActD is not showing a distinct effect between Mdm2 WT and 6A, since several publications have shown that ActD treatment can lead to S15 phosphorylation of p53. In accordance with lower p21 induction, the cell cycle arrest as measured by H3-Thymidine incorporation is delayed in the 6A U2OS cells. What surprises me somewhat is that at 24 hrs the difference between WT and 6A has almost disappeared, while in figure 7 still at 24 hrs no induction of p21 is seen. Is this a p53/p21-independent effect upon IR?

Response: Unlike gamma irradiation that only lasts a few minutes, the NCS experiment was a continuous treatment to induce persistent DNA damage. This is likely to cause other toxicity that reduces p21 expression.
We used actinomycin D at 5 nM which acts by inhibiting rRNA metabolism and causing nucleolar stress without DNA damage. Most studies use 10-fold or higher concentrations that causes DNA damage. This distinction has been documented by studies from Karen Vousden and Yanping Zhang labs.
IR induces a delayed G2/M arrest in U2OS cells that is p53-independent. Only the early G1 arrest is p53-dependent. Therefore the lack of difference at 24 hr is expected.

It should be mentioned that the fact that phosphorylation of Mdm2 by an ATM-dependent manner is needed for full p53 activation has been shown earlier quite clearly by Stommel and Wahl, so the experiments shown in figures 7 and 8 are not really providing more insight into regulation of p53-Mdm2 upon DNA damage.

Response: We agree that the role of ATM in p53 activation is well established. Our data here illustrates the importance of the MDM2 phosphorylation sites for this regulation, which has not been shown before.

The authors argue in the Discussion section that they do not see a transient reduction of Mdm2 levels upon IR, but that this original observation might be an artifact of the use of the SMP14 antibody. Although possibly outside of the scope of this manuscript, this is an important observation that would shed new light on earlier publications. Therefore, either the authors should show this distinct effect of SMP14 (with the use of the parental U2OS cells, for example), or it would be better not to mention it at all.

Response: As suggested, we removed the comment on SMP14. This is an important issue and will be addressed elsewhere.

Minor/specific comments:
Figure 1C: typo in labeling (it says PS4386). In legends it says that cells are treated with 10 Gy for 4 hrs. I think that is not completely correct. Cells are irradiated with 10 Gy, and after 4 hrs they are harvested. Similar descriptions are found elsewhere in the legends.

Response: We have made corrections in the text.

Figure 2A: what is the meaning of the His6-Mdm2 in this figure/experiment? Either mention it somewhere or leave it out.

Response: We use His6-MDM2 to verify phospho antibody specificity, since it is produced in E.

coli and should not react with phospho antibodies.

Figure 2C: I would have expected that 2 hrs after IR levels of p53 and Mdm2 are already increased to some extent, based also upon figure 2a. Why is that not seen here? Or, were the cells treated with MG132?

Response: MG132 was used to normalize protein levels. Clarification is added to legend.

Figure 3a: the authors say that the phosphor-mimicking mutants of Mdm2 are 'deficient' in degradation of p53. Maybe this is a bit too strongly put, since they clearly show still quite an activity. It would be nice if the authors would have quantified figure 3a, or even better would have shown a titration experiment with the mutants and wt side-by-side.

Response: We have modified the text and added a titration experiment as suggested (Figure 3c).

As mentioned above, an experiment as shown in figure 4a should be repeated, and probed not only with anti-Myc but also with anti-p53. Theoretically the last incubation should give a result as in the His-Ub pull-down, but the whole experiment is somewhat different, so should be tested.

Response: We have added the experiment as suggested in new Figure 4a.

Supplemental figure 1: Self-Ub of Mdm2: I would say that the 6D is more active than wild-type. Is this seen consistently?

Response: When the level of 6D is taken into consideration, there is no real difference in self ubiquitination level. We had looked extensively at the possibility of 6D affecting self ubiquitination and self degradation but found no real changes.

Figure 6: the 4B11 antibody has its epitope in the RING domain. Is the excluded that phosphorylation of the RING on one or more sites could weaken the interaction with 4B11, so that actually the amount of P-RING is underestimated?

Response: 4B11 reactivity is not affected by phosphorylation. Figure 6b shows that the efficiency of MDM2 phosphorylation can approach 100% after IR, based on complete shifting of RING domain mobility.

Referee #3 (Remarks to the Author):

In their manuscript, the authors suggest that ATM stabilizes p53 in response to IR by phosphorylating several amino acids in the C-terminal part of Mdm2 which reduce Mdm2 oligomerization via the Ring domain.
To support their conclusions, the authors showed that two (S386; S429) of 6 phosphorylation sites identified by mass spec are phosphorylated in vivo by using phosphorylation specific antibodies. ATM can phosphorylate these sites in vitro and phosphorylation is absent in ATM-negative cells or after ATM inhibition. Replacement of the phosphorylated serines with acidic amino acids prevented p53 degradation. While replacing the 6 terminal serines with an alanine enhanced p53 ubiqutiylation, and reduced the accumulation of p53 after IR after ectopic expression, substituting them with an aspartic acid prevented p53 ubiquitylation. The authors furthermore argue that phosphorylation of the C-terminus of Mdm2 prevents RING domain oligomerization.

This is indeed an elegant study with a number of clever experiments to resolve a complex issue. However, sophisticated experiments also bear the intrinsic risk of creating artificial models and the regulation of p53 appears to be particularly prone to artefacts due to results of cell culture experiments that cannot be reproduced e.g. in animal models. Therefore, these studies need to be evaluated particularly carefully.

Intriguingly, the results from Chen at al., as beautiful and consistent as they are, do not not comply with several previous and important observations.
1) Lu and Lane reported in 1993 that the kinetics of p53 in response to UV and ionising irradiation

differs, a finding that has been reproduced by several investigators in the meantime. Cheng et al., though, show that serine 386 and serine 429 of Mdm2 are phosphorylated at the same time and to the same extend (Figure 1d), suggesting that phosphorylation of these sites does not differe between UV and IR.

Response: In this paper we did not focus on the kinetics of UV response. Figure 1d only analyzed a single 4 hr time point with high dose UV. So our result really does not say much about the difference between IR and UV. Given the high UV dose used here, MDM2 phosphorylation is expected.

2) Maki and Howley showed in 1997 that in contrast to UV-irradiation, p53 that accumulates after IR is still ubiquitylated, a finding that is, moreover, consistent with the different kinetics of p53 accumulation that are most likely due to different mechanisms. In contrast, Chen et al., report that ATM phosphorylation of Mdm2 prevents p53 polyubiquitylation.

Response: We are well aware of the 1997 MCB paper by Maki and Howley. The experiments in the paper did not normalize the level of p53 loading after IR, therefore it did not specifically address whether the ratio of p53-ubiquitin/total p53 is changed after IR. In fact, if the large increase in p53 level after IR in their experiments were taken into consideration, one would conclude that IR reduces p53 ubiquitination.

3) Boehme et al., have shown in 2008 that p53 accumulates independent of ATM, at least in some cell lines. Cheng et al., though suggest that the accumulation of p53 in response to Mdm2-RING phosphorylation is caused by the ATM kinase.
Since these previous observations have been derived from non-manipulated cells they do not bear such a high risk of false results as observations that are derived upon manipulating the system by transfections and mutagenesis. These issues therefore need to be carefully addressed.

Response: The important role of ATM in IR-induced p53 stabilization has been extensively reported in the literature, including our own published data using human skin fibroblasts. Boehme et al. reported ATM-independent p53 response in lymphoblasts. The authors themselves suggested that it may be due to cell type specificity. In fact, in their study, ATM knockdown in U2OS cells also reduced p53 IR response.

Additional comments:
- *1) figure 3a: what is the reason why one serine was replaced with aspartic acid while the other one was replaced with glutamic acid? How would the result look if serine 386 would be replaced with aspartic acid and serine 429 with glutamic acid? Nevertheless, it needs to be kept in mind that replacing a serine with an acidic acid does not always mimic a phosphorylated serine.*

Response: Mutating the 386S codon (TCA) to a D (GAT or GAC) would require changing all 3 bases, therefore a 2 bp change to E (GAA) was chosen. We agree that D or E substitutions are not ideal mimics of phosphorylation, for example they do not function as potential docking sites for 14-3-3. However, if the mechanism is mainly due to negative charge from the phosphate, D or E will be a weak mimic of phosphate by adding 1 negative charge instead of 3. As far as we know, both residues work well in some context, but may be different in others.

- *2) figure 3b) Obviously, the Mdm2 mutant Mdm2-6A is more competent than wild type Mdm2 in targeting for p53 degradation. Since wild type Mdm2 was not phosphorylated in the C-terminus under normal conditions as shown for example in figure 1b, 2c or 2c, this could indicate that replacing 6 amino acids with an alanine has an impact on Mdm2 activity that is distinct from phosphorylation of these serines.*

Response: Figure 1b shows that there is basal phosphorylation of S386 in normal conditions. We also found that transient transfected MDM2 has basal phosphorylation of MDM2 (Fig 1c), which explains the hyperactivity of 6A in such assays because it is resistant to basal modification. The fact that CIP treatment of Wt MDM2 stimulates its activity to the same as 6A (Figure 4b) also indicates the presence of basal phosphorylation, and suggests that 6A mainly affects phosphorylation, not by other effects.

- *3) figure 4a) This figure shows that the 6D mutant does no longer polyubiquitylate p53. In order to distinguish between non- and mono-ubiqutiylation, it is essential to include a sample where no ubiquitin has been transfected to determine background levels.*

Response: The suggested control is included in the revised Figure 4.

- *4) figure 4b) Strikingly, while in figure 4a, the 6D mutant lacked any E3 activity, in figure 4b, and in supplementary figure 2a, the 6D mutant is almost as active as wild type Mdm2 in ubiquitylating p53?*

Response: As discussed above in response to Reviewer 2, the 6D mutant was mainly defective in p53 poly ubiquitination as shown in Myc-ubiquitin assay (Figure 4a) and in vitro ubiquitination assay (Figure 4b), but retains significant activity in p53 mono ubiquitination (Figure 4, S2a). The differences between these assays are discussed above.

- *4) figure 6c) Although most likely, there is no formal proof in this figure hat the smaller forms are indeed phosphorylated Mdm2. In addition, there is also a shift in the early fraction upon treatment with CIP.*

Response: Revised Figure 6c using a lighter exposure shows that CIP did not affect early fraction mobility.

- *5) figure 5 and Supplementary figure 3) The gel filtration experiments look vary variable. While in figure 5c, wt C-term Mdm2 fractionates as oligomers and monomers, in supplementary figure 3a, Mdm2 is everywhere, although the reviewer agrees that the concentration is higher in earlier fractions. In contrast, in supplementary figure 3b, , Mdm2 is only in the early fractions. Taken all these different experiments into account, the 6D mutant in supplementary figure 3b did not show a clear difference to the wild type protein in supplemental figure 3a or to the Ku55933-treated and irradiated wild type Mdm2 in figure 5c. Moreover, while the single substituted serine 386 aspartic acid mutant, showed a clear difference to wild type Mdm2 in gel filtration experiments (supplementary figure 3a), the corresponding alanine mutant had no effect on p53 degradation as shown in supplementary figure 4.*

Response: In Figure S3a, Wt MDM2 was from transiently transfected H1299 cells whereas Figure 5c MDM2 was from endogenous SJSA cells at higher levels. This may have affected their behavior on gel filtration.
The comment on GST-MDM2-6D is similar to Reviewer 1 and 2 and has been addressed above. We repeated the gel filtration of GST-MDM2-WT and GST-MDM2-6D using much lower level (similar to cell culture shown in Figure 5C) and there is a significant difference between WT and 6D (new Figure S3b).
Our results indicate that the phosphorylation sites are redundant. Therefore, blocking phosphorylation at a single site with alanine substitution is not expected to have an effect because other phosphorylation sites can be modified. Whereas a single phosphorylation mimic is sufficient to have strong effect.

- *6) supplementary figure 4: Obviously, only replacement of all 6 C-terminal serines affects Mdm2 activity, but only phosphorylation of three of them (S 386, S395, S429) is supported by additional experiments, e.g. by phosphorylation-specific experiments. Moreover, alanine replacement of these 3 serines, that have been clearly shown to be phosphorylated after IR, does not impair Mdm2 function. How sure can we be that the 6A and 6D mutants really mimick the physiologic situation?*

Response: The triple alanine mutant not being able to prevent p53 response suggest that the 6 sites are redundant and all need to be blocked to prevent signaling. Leaving a single site to be phosphorylated will activate p53. Therefore, 6A creates an ATM-deficient effect for MDM2, and 6D is similar to 386E that is already quite inactive.
The current mass spect technology cannot yet tell whether all sites are phosphorylated at the same time on a single MDM2 molecule. But since the sites are redundant, a single phosphorylation event is already a strong signal to p53. Whether all 6 sites are modified on a single MDM2 becomes less important.

- *7) supplementary figure 4b) How does it come that p53 levels are high at 0hr after transfection of cDNA3 and Mdm2, lower at 3hr and particularly low at 6hr after irradiation?*

Response: This figure used normalized p53 loading in order to clearly show the changes in acetylation/p53 ratio. We found that the increase in p53 level after IR makes the result hard to interpret without compensating loading for the untreated sample. A clarification is added to the legend.

We hope the new results and modifications have addressed the concerns of the reviewers. We look forward to a quick review.

2nd Revision - authors' response 20 August 2009

Thank you for submitting your revised manuscript for consideration by The EMBO Journal, and apologies for the delay caused by referee inavailability at this time of the year. We have now eventually heard back from one of the original reviewers, whose comments are copied below. On the whole, this referee feels that most major comments of the original round of review have been satisfactorily addressed and that the paper is thus significantly improved. In this light, we should in principle be able to go ahead with publication, but there are nevertheless a few remaining issued detailed by the reviewer that would require further attention before the study should be ready for acceptance. While I note that the minor comments of referee 2, and also some of his/her major issues can probably be clarified without further experimentation, there are also still some points that would need further data, especially the well-taken point that a direct (p53-independent) effect of Mdm2 mutants on p21 stability can currently not be ruled out in a sufficiently decisive manner.

In this light, I am therefore returning the study to you once more for an exceptional extra round of revision here, in which I hope you will be able to satisfactorily deal with the referee's remaining concerns and comments. Therefore, please get a re-revised version of the manuscript including a response letter back to us as soon as possible. Of course, please feel free to contact me if there is anything to be clarified further before submission of this final revision.

I am looking forward to receiving your final version.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The revised manuscript by Cheng et al., 'ATM activates p53 by regulating MDM2 oligomerization en E3 processivity', has significantly increased in clarity and quality.

As in the original version, the initial data/figures are clear and show the existence of multiple ATM-dependent phosphorylation sites in the Mdm2 RING.
With the use of mutants in which one or more of the six phosphorylation sites are altered into phospho-mimicking amino acids, it was found that mutating just one of these sites is sufficient to inhibit the degradation of p53 by Mdm2. Mutating all does not appear to have an additional effect. On the other hand, such mutations do not appear to affect the degradation of Mdmx by Mdm2, and also do not apparently affect the ubiquitination of Mdm2 itself.
Subsequent data suggest that the phosphorylation of the RING inhibits the Mdm2 oligomerization, and that monomeric Mdm2 can only trigger (multiple-) mono-Ub of p53. Although some figures are still not very clear, and I still have some minor comments/suggestions, in general these conclusions

are based upon rather well controlled experiments.
In my original review I wrote the following: "Even so, assuming that indeed the phosphor-Mdm2 RING cannot homo-dimerize, it still can bind to the RING of Mdmx. Based upon publications from the Vousden and the Yuan lab, one would expect that such a heterodimer would be perfectly able to ubiquitinate and degrade p53, maybe even better than an Mdm2 homo-oligomer. I am very surprised that he authors not even discuss these publications." The answer of the authors is that Linke et al. showed that MDM2-MDMX heterodimer has much weaker E3 activity compared to MDM2 homodimer in vitro. Actually, I think these authors do not stress that point, but that their main conclusion is, as is in the title, that "dimerization is required for their ubiquitylation in trans". Suggesting that a monomer of Mdm2 has not significant 'self'ubiquitination.
Regarding the experiments with the stable U2OS cell lines: the author shave clarified some points, but I still would like to see additional figures. First, why not direct put lysates of the three U2OS cell lines side-by-side to show the levels of various proteins, maybe in comparison even with SJSA cells. Very easy, and would be very clarifying. Secondly, although indeed in the MDM2-6A cell line the levels of p21 are not/hardly induced, it is not shown that this is caused by the reduced stabilization/activation of p53. As I mentioned earlier, MDM2 has been suggested to directly Ub and degrade p21; theoretically there could be a difference in that regulation between Mdm2-WT and Mdm2-6A. In addition, only investigating one p53 single target protein is limited. I would very much like to see some real-time PCR experiments for at least two different p53 target genes, e.g. p21 and PUMA.
Also the response of my comments on figure 7b is not relevant. Of course NCS is always on the cells and IR is a short treatment, but that difference does not explain why the cDNA3 and the MDM2-WT cell lines respond apparently differently to NCS regarding p21 induction, although p53 induction appears to be similar. Basal p53 levels in the U2OS-6A cell line is lower in this exposure; try to show a better exposure so that comparison can be better made. Furthermore, I would have preferred to see a longer time-course, also with NCS like in the IR experiment.

Minor comments:
- I would say there still is a discrepancy between Figure 4a and 4b: authors argue in 4b that there is not too much difference between Mdm2 WT and Mdm2-6D, because of 'basal' phosphorylation upon transfection. However, in figure 4a strong difference between Mdm2-WT and MDM2-6A. Why?
- it would have been nice if the authors would have used also anti-phospho S386 on gel filtration figures 5c.

- I wonder whether the authors have also used the Mdm2-6A mutant in their oligomerization studies. Does this mutant only form oligomers?

2nd Revision - authors' response 04 September 2009

We are submitting a revised manuscript titled "ATM activates p53 by regulating MDM2 oligomerization and E3 processivity" to the EMBO Journal. The manuscript had been reviewed as EMBOJ-2009-71449R. We have now added additional data in response to the remaining comments of Reviewer 2.

Major changes in figures

Supplemental Figure S4c is added that has all 3 cell lines run on the same gel to facilitate comparison of basal p53 and p21 levels.

Supplemental Figure S4a is added to show the quantitation of unstressed MDM2 levels in the U2OS stable cell lines compared to SJSA.

A Referee-only supplemental figure is added regard p21 degradation by MDM2 mutants.

Referee #2 (Remarks to the Author):

In my original review I wrote the following: "Even so, assuming that indeed the phosphor-Mdm2 RING cannot homo-dimerize, it still can bind to the RING of Mdmx. Based upon publications from the Vousden and the Yuan lab, one would expect that such a heterodimer would be perfectly able to ubiquitinate and degrade p53, maybe even better than an Mdm2 homo-oligomer. I am very surprised that he authors not even discuss these publications." The answer of the authors is that Linke et al. showed that MDM2-MDMX heterodimer has much weaker E3 activity compared to MDM2 homodimer in vitro. Actually, I think these authors do not stress that point, but that their main conclusion is, as is in the title, that "dimerization is required for their ubiquitylation in trans". Suggesting that a monomer of Mdm2 has not significant 'self'ubiquitination.

Response: Although the main focus of the Linke study was on MDM2-MDMX RING co-crystal structure, their data actually contains direct biochemical evidence that MDM2-MDMX dimer is less active than MDM2-MDM2 dimer. Other cell culture studies in the literature show that MDMX can increase p53 degradation when MDM2 is at sub-optimal levels (Yuan study) or functionally deficient due to C terminal mutation (Vousden study). They did not compare the relative activity of MDM2-MDMX heterodimer and MDM2-MDM2 homo dimer. The stimulatory effect of MDMX seen in those studies were due to the ability of MDMX to complement sub-threshold MDM2 or mutant MDM2 in forming active hetero dimer, but the Linke study shows that hetero dimers are much less active than MDM2 homo dimer.
The reviewer's point is that phospho-MDM2 should still be as active as wild type MDM2 because it can still bind MDMX. However, what happens in the cell is that after DNA damage, MDMX is rapidly degraded due to phosphorylation. Therefore, hetero dimer formation will have insufficient quantity and activity to prevent p53 activation after DNA damage.

Regarding the experiments with the stable U2OS cell lines: the author shave clarified some points, but I still would like to see additional figures. First, why not direct put lysates of the three U2OS cell lines side-by-side to show the levels of various proteins, maybe in comparison even with SJSA cells. Very easy, and would be very clarifying.

Response: Supplemental Figure S4c is added that has all 3 cell lines run on the same gel for comparison. It shows that MDM2-Wt and MDM2-6A cells have slightly reduced basal levels of p53 and p21 in the absence of DNA damage, consistent with Figure 7a. A new Supplemental Figure S4a is added to show the quantitation of unstressed MDM2 levels in the cell lines compared to SJSA.

Secondly, although indeed in the MDM2-6A cell line the levels of p21 are not/hardly induced, it is not shown that this is caused by the reduced stabilization/activation of p53. As I mentioned earlier, MDM2 has been suggested to directly Ub and degrade p21; theoretically there could be a difference in that regulation between Mdm2-WT and Mdm2-6A. In addition, only investigating one p53 single target protein is limited. I would very much like to see some real-time PCR experiments for at least two different p53 target genes, e.g. p21 and PUMA.

Response: It was reported that MDM2 degrades p21 through ubiquitin-independent mechanism. However, our lab had not been able to convincingly reproduce this finding several years ago. Upon request by the reviewer, we carefully repeated the experiment several times by transfecting different doses of p21 with MDM2/6A/6D and still did not notice a clear degradation of p21. Therefore, our results suggest that p21 degradation by MDM2 is a weak phenotype at best, and MDM2-6A does not have increased p21 degradation efficiency. A referee-only figure is provided as supplemental material.
Previous studies (including the 2003 EMBO J paper from Hua Lu) have specifically determined that MDM2 does not affect p21 mRNA level. Therefore, the lack of p21 induction in 6A cells is most likely due to lack of p53 accumulation.

Also the response of my comments on figure 7b is not relevant. Of course NCS is always on the cells and IR is a short treatment, but that difference does not explain why the cDNA3 and the MDM2-WT cell lines respond apparently differently to NCS regarding p21 induction, although p53 induction appears to be similar. Basal p53 levels in the U2OS-6A cell line is lower in this exposure; try to show a better exposure so that comparison can be better made. Furthermore, I would have preferred to see a longer time-course, also with NCS like in the IR experiment.

Response: In MDM2-Wt cells, a fraction of the stabilized p53 is sequestered by high level MDM2 after NCS treatment (despite p53 phosphorylation), therefore p21 induction will not be as efficient as free p53 in cDNA3 control cells. In fact, Figure 7a also shows reduced p21 induction in MDM2-Wt cells after IR due to the same reason. Therefore NCS and IR effects are similar.

Minor comments:
- I would say there still is a discrepancy between Figure 4a and 4b: authors argue in 4b that there is not too much difference between Mdm2 WT and Mdm2-6D, because of 'basal' phosphorylation upon transfection. However, in figure 4a strong difference between Mdm2-WT and MDM2-6A. Why?
- it would have been nice if the authors would have used also anti-phospho S386 on gel filtration figures 5c.
- I wonder whether the authors have also used the Mdm2-6A mutant in their oligomerization studies. Does this mutant only form oligomers?

Response: A strong difference between MDM2-Wt and MDM2-6A is expected since 6A is resistant to basal phosphorylation and remains fully active, while MDM2-Wt is partially inactivated by basal phosphorylation. Therefore, the results are consistent with the activity ranking of 6A>>Wt>6D in terms of p53 poly ubiquitination.

The amount of MDM2 from gel filtration fractions was too low for analysis by phospho antibodies despite our effort. The question was indirectly addressed by results in Figure 6b/6c. We performed gel filtration analysis of U2OS-MDM2-6A under non-stressed condition and the result showed absence of monomeric form, consistent with the results of ATM inhibitor treated SJSA cells.

We hope the new results and modifications have addressed the remaining concerns of the reviewer and made the manuscript suitable for publication. We look forward to a quick decision.

